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AUTHOR(S):

Ogawa, Toshihiro; Akazawa, Toshiyuki; Tabata, Yasuhiko

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***In Vitro* Proliferation and Chondrogenic Differentiation of Rat Bone Marrow Stem Cells Cultured with Gelatin Hydrogel Microspheres for TGF- β 1 Release**

Toshihiro Ogawa^a, Toshiyuki Akazawa^b and Yasuhiko Tabata^{a,*}

^a Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

^b Hokkaido Industrial Research Institute, Nishi-11, Kita-19, Kita-ku, Sapporo, 060-0819, Japan

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Abstract

The objective of this study was to evaluate the proliferation and chondrogenic differentiation of rat bone marrow-derived mesenchymal stem cells (MSCs) cultured with gelatin hydrogel microspheres of cell scaffold which can release transforming growth factor- β 1 (TGF- β 1). Gelatin was dehydrothermally cross-linked in different conditions in a water-in-oil emulsion state to obtain gelatin hydrogel microspheres with different water content. The microspheres functioned not only as the scaffold of MSC, but also the carrier matrix of TGF- β 1 release. The MSC proliferation depended on the water content of microspheres. Higher MSC proliferation was observed for the gelatin microspheres with lower water content. When cultured with the gelatin hydrogel microspheres, MSC formed their aggregates, in contrast to culturing with hydrogel sheets. The cell viability was significantly high compared with that of the hydrogel sheet. The production of sulfated glycosaminoglycan (sGAG) from MSC was examined as a measure of chondrogenic differentiation, after their culturing in a normal and chondrogenic differentiation media. For both the cultures, the amount of sGAG produced was significantly higher for MSC cultured with the gelatin microspheres than that of the gelatin sheet. Stronger differentiation of MSC was achieved in culture with the microspheres incorporating TGF- β 1 than that of MSC cultured in the medium containing the same amount of TGF- β 1. It is concluded that the gelatin hydrogel microspheres function well as both the scaffold of MSC and the matrix of TGF- β 1 release, resulting in enhanced MSC aggregation and the consequent promotion of cell proliferation and differentiation.

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Keywords

MSC, proliferation, chondrogenic differentiation, TGF- β 1 release, microspheres scaffold

* To whom correspondence should be addressed. Tel.: (81-75) 751-4121; Fax: (81-75) 751-4646; e-mail: yasuhiko@frontier.kyoto-u.ac.jp

1. Introduction

For cell-based tissue regeneration, it is necessary to combine cells and their local environment for their promotion of biological activity. Stem cells have been noted as the cell source of tissue-regeneration therapy because of their high potential of proliferation and differentiation. Bone marrow mesenchymal stem cells (MSCs) are now being used in clinical therapy [1–4] and can differentiate into different cell lineages, such as bone, cartilage and muscle cells. As the environment, various cell scaffolds are designed and prepared from biomaterials [1–4]. The sponge and non-woven fabrics of polymers have been experimentally investigated to demonstrate the necessity and feasibility as the scaffold of cell proliferation and differentiation [5, 6]. Undoubtedly, the scaffold needs the initial attachment of cells and the subsequent proliferation. However, when present for a long time the scaffold often causes inflammatory responses which may impair the process of tissue regeneration. One trial to tackle the problem is to accelerate the biodegradation of scaffold material. Another is to reduce the amount of scaffold material. To this end, a microsphere and nanofiber scaffold has been used [5, 8].

Microspheres have been widely used for the suspension culture of cells to produce human recombinant proteins and peptides of therapeutic activity in biotechnology [6]. Since the microspheres have a large specific surface to volume, they are suitable as the substrate of cell culture. However, little has been applied to the cell scaffold of tissue engineering. Different from the microsphere substrate for biotechnology, the material of cell scaffold should be degraded and compatible to cells. In addition, it is preferable to have a property that the scaffold can release the growth factor to accelerate cell proliferation. There have been reported on the controlled release of growth factor from various materials [7–9]. From the viewpoint of tissue regeneration, the material for factor release must be degraded to disappear when the factor release is completed. The material remaining often causes physical hindrance of tissue regeneration. We have demonstrated that the hydrogel of gelatin has an ability to release various growth factors and promote growth factor-induced tissue regeneration [9]. The growth factor is released as a result of biodegradation of gelatin hydrogel. For this hydrogel release system, the problem of material remaining is overcome. In addition, the gelatin hydrogel is cytocompatible and biodegradable. Based on these findings, gelatin was selected as the material of cell scaffold.

This study was undertaken to obtain fundamental information about the proliferation and differentiation of MSCs cultured with the cell scaffold of microsphere type. MSCs were isolated from the bone marrow of rats and cultured with biodegradable microspheres of gelatin hydrogel. MSC proliferation and production of sulfated-glycosaminoglycan (sGAG), a measure of chondrogenic differentiation, were evaluated and compared with those cultured with the sheet of gelatin hydrogels. In addition to the role as cell scaffold, the gelatin hydrogel microspheres can function as the carrier matrix of transforming growth factor- β 1 (TGF- β 1) release.

We examined the effect of TGF- β 1 released from the microspheres on the sGAG production.

2. Materials and Methods

2.1. Preparation of Gelatin Hydrogel Microspheres

Gelatin microspheres were prepared by the chemical cross-linking of gelatin in a water-in-oil emulsion state according to the method previously reported [10]. Briefly, an aqueous solution (20 ml) of 10 wt% gelatin (isoelectric point 5.0, weight-average molecular weight 1×10^5 ; Nitta Gelatin, Osaka, Japan) was preheated at 40°C, and then added dropwise into 600 ml olive oil at 40°C, followed by stirring at 200 rpm for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4°C for the natural gelation of gelatin solution to obtain non-cross-linked microspheres. The resulting microspheres were washed with cold acetone and centrifugated (5000 rpm, 4°C, 5 min) three times to exclude residual oil completely. Then, they were fractionated in size by sieves with apertures of 75 and 125 μ m and air-dried at 4°C. The non-cross-linked and dried gelatin microspheres (200 mg) were treated in a vacuum oven at 140°C and 0.1 Torr for dehydrothermal cross-linking of gelatin according to the method reported previously [11]. The microscopic photographs of gelatin hydrogel microspheres in the dried or water-swollen state were taken to measure their size (400 microspheres per each sample). Based on the volume of microspheres calculated from the size, the water content of microspheres, which is defined as the volume percentage of water to gelatin hydrogel microspheres swollen, was calculated.

2.2. Preparation of Gelatin Hydrogel Microspheres Incorporating TGF- β 1

Powdered TGF- β 1 (R&D Systems, Minneapolis, MN, USA) was dissolved in double-distilled water (DDW) to give a solution at the TGF- β 1 concentration at 1 μ g/ml. The TGF- β 1 solution (10 μ l) was dropped onto 1 mg of freeze-dried gelatin hydrogel microspheres, followed by leaving at 25°C for 1 h for impregnation of TGF- β 1 into the microspheres. The TGF- β 1 solution was completely absorbed into the microspheres through the impregnation process because the solution volume was much less than that theoretically required for the equilibrated swelling of microspheres.

2.3. Evaluation of TGF- β 1 Release from Gelatin Hydrogel Microspheres Incorporating TGF- β 1

A suspension (1 ml) of gelatin hydrogel microspheres incorporating 10 ng TGF- β 1 in 10 mM phosphate-buffered saline (PBS, pH 7.4) was transferred into a tube, followed by stirring at 37°C. At different time intervals, the suspension was centrifuged (5000 rpm, 5 min) and the supernatant was completely collected and replaced with fresh PBS. The amount of TGF- β 1 released was measured by enzyme-linked immunosorbent assay (ELISA) with a commercially available kit

(R&D Systems) according to the manufacturer's instructions. The release test was done in triplicates for each sampling time.

2.4. Preparation of Gelatin Hydrogel Sheets

An aqueous solution of 10 wt% gelatin (5 ml) was cast into a polypropylene mold ($2 \times 2 \text{ cm}^2$) and left at 4°C for 24 h for gelation, followed by freeze-drying. The resulting gelatin hydrogel sheets freeze-dried were cross-linked by dehydrothermal treatment at 140°C for 18 h in a vacuum condition. The hydrogel sheets were swollen in DDW at 37°C for 24 h until to the equilibrium. The gelatin hydrogel sheets were stored in DDW at 4°C until to use or assay [14].

2.5. Preparation of MSC

MSC were prepared from the bone marrow of 3-week-old male Wister rats (Shimizu Laboratory Supply, Kyoto, Japan). Briefly, both ends of rat femurs were cut away from the epiphysis, and the bone marrow was flushed out using a syringe (21 gauge needle) with 1 ml PBS. The cell suspension was placed into two 25 cm^2 flasks (Iwaki Glass, Funabashi, Chiba, Japan) with 4 ml alpha minimum essential medium (α MEM) supplemented with 15 vol% fetal calf serum (FCS), penicillin (50 U/ml), and streptomycin (50 U/ml) (standard medium) and cultured at 37°C in a 95% air/5% CO_2 atmosphere. The medium was exchanged on day 3 after culture and every 3 days thereafter. When became sub-confluent, 7–10 days of culture later, the cells were detached by treatment for 5 min at 37°C with PBS containing 0.25 wt% trypsin and 0.02 wt% ethylenediaminetetraacetic acid. The cells were subcultured at a density of $2 \times 10^4 \text{ cells/cm}^2$. When proliferated to reach a sub-confluence state, cells were used for the following experiments.

2.6. MSC Culture with Gelatin Hydrogel Microspheres

MSCs ($1 \times 10^5 \text{ cells/ml}$) and the gelatin hydrogel microspheres (1 mg/ml) were suspended in the standard medium and added into each well of a 48-well multi-dish culture plate (Code 1830-048, Iwaki brand, Scitech Div. Asahi Techno Glass, Chiba, Japan) at a volume of 1 ml/well, followed by incubation for 24 h at 37°C to allow cells to attach on the microspheres' surface. Then, the MSC-attached microspheres were transferred into each well of 48- and 6-well multi-dish culture plate and cultured in 1 ml of medium for 1, 4, 7, 10 and 14 days. The medium was changed on day 3, 6, 9, 11 and 13. As control, MSCs were cultured on each well of a 48-well multi-dish culture plate or the gelatin hydrogel sheet at a density $1 \times 10^5 \text{ cells/well}$.

2.7. Cell Number Measurement

The number of cells proliferated was determined by the fluorometric DNA assay (Rao and Otto). Briefly, cells with or without the hydrogel microspheres were washed with PBS 3 times and stored at -30°C until assay. After thawing, the cells were digested in 30 mM sodium citrate-buffered saline solution (SSC, pH 7.4) containing proteinase K (250 $\mu\text{g/ml}$) and sodium dodecyl sulfate (0.2 mg/ml) at 65°C

for 12 h. After the cell sample solution (100 μ l) was mixed with 100 μ l SSC containing 1 mg/ml Hoechst 33258 dye, the fluorescence intensity of the mixed solution was measured in a fluorescence spectrometer (Spectra Max Gemini EM, Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 355 and 460 nm, respectively. The cell number was obtained using the calibration curve which had been prepared with cell suspension at determined cell number. Three wells were used for each sample, unless otherwise mentioned.

2.8. Cell Viability

Cell viability was assayed using a living cell counting kit (Nacalai Tesque, Kyoto, Japan). A solution of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) (100 μ l) was added into each well of cells cultured for 1, 4, 7, 10 and 14 days, followed by additional incubation for 3 h. The absorbance of samples was measured at 450 nm using a VERSAmax microplate reader (Molecular Devices).

2.9. Differentiation Evaluation of MSC Cultured with Gelatin Hydrogel Microspheres

Chondrogenic differentiation medium used was the standard medium containing 100 μ g/ml sodium pyruvate (Lonza, Tokyo, Japan), 100 nM dexamethasone, $1 \times$ Insulin-Transferrin-Selenium-X (ITS+premix), 40 μ g/ml proline and 25 μ g/ml ascorbate-2-phosphate (Sigma, Poole, UK). Cell culture was performed in a similar way as described above, but with another differentiation medium. To evaluate the effect of TGF- β 1 on the cell differentiation, TGF- β 1 was added to the cell culture in the following three ways while the total amount of TGF- β 1 added was 10 ng; addition of 10 ng/ml TGF- β 1 once on day 0, 1.25 ng/ml TGF- β 1 8 times on day 0, 2, 4, 6, 8, 10, 12 and 14, and microspheres containing 10 ng TGF- β 1 once. To prepare gelatin hydrogel microspheres incorporating TGF- β 1, 10 μ l PBS containing 10 ng TGF- β 1 was dropped onto 1 mg freeze-dried gelatin microspheres, followed by leaving for 1 h at 37°C to allow the solution to adsorb into the microspheres. The similar procedure other than using PBS without TGF- β 1 was done to prepare TGF- β 1-free gelatin hydrogel microspheres.

2.10. sGAG Assay

The amount of sGAG produced from MSCs cultured with gelatin hydrogel microspheres with or without TGF- β 1 incorporation was determined by the dimethyl-methylene blue dye (DMMB) assay previously reported [12]. The cell sample solution prepared (30 μ l) by the method described above was mixed with 270 μ l of DMMB solution. Then, the solution absorbance was measured at 525 nm using a VERSAmax microplate reader (Molecular Devices). The content of sGAG produced from the cells cultured in different manners was calculated by a curve prepared by use of known amounts of chondroitin sulfate.

3. Results

3.1. Characterization of Gelatin Hydrogel Microspheres

Table 1 summarizes the size and water content of gelatin hydrogel microspheres prepared in different conditions. Figure 1 shows the typical microscopic photograph of gelatin hydrogel microspheres. The microspheres were of spherical shape and had a smooth surface. The size of microspheres was around 250 μm , irrespective of the cross-linking condition. The water content of microspheres, which corresponded well to the extent of cross-linking, decreased with an increase in the treatment time for dehydrothermal cross-linking. The cross-linking extent of microspheres is increased by the treatment time and consequently their water content is decreased.

Figure 2 shows the time profile of TGF- β 1 release from gelatin hydrogel microspheres in PBS. The biological activity of TGF- β 1 was released with time from the microspheres. 15% of TGF- β 1 was incorporated over 2 weeks, most TGF- β 1 remained un-released in the microspheres.

Table 1.
Preparation and characterization of gelatin hydrogel microspheres by dehydrothermal cross-linking for different periods of time at 140°C

Time (h)	Microsphere size (μm)	Water content (vol%)
0	n.m.	n.m.
12	439 \pm 234	98.7 \pm 0.69
24	153 \pm 77	96.3 \pm 1.00
48	107 \pm 35	93.5 \pm 0.95
72	114 \pm 41	92.4 \pm 1.10

Values are mean \pm SD. n.m., not measurable.

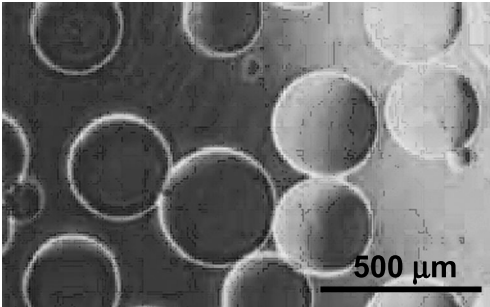


Figure 1. A microscopic photograph of gelatin hydrogel microspheres with a water content of 93.5 wt% dispensed in water.

3.2. Proliferation of MSC Cultured with Gelatin Hydrogel Microspheres and Sheets

Figure 3 shows the time profile of MSC proliferation after incubation with gelatin hydrogel microspheres with different water content. For the microspheres with lower water contents, cells proliferated at higher rates than those of higher water content microspheres. The cell number decreased rapidly 7 days after culturing in the case of microspheres with higher water content. The cells tended to detach from the surface of microspheres.

Figure 4 shows the MSC proliferation in culture with the gelatin microspheres at different densities. For a high density culture in 48-well multi-dish, cells were

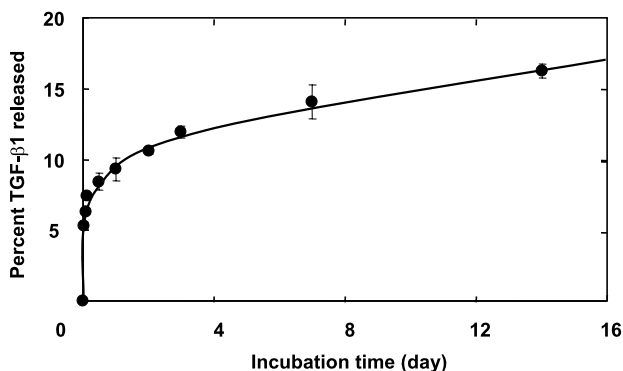


Figure 2. Time profile of *in vitro* TGF-β1 release from gelatin hydrogel microspheres incorporating TGF-β1 with a water content of 93.5 wt%.

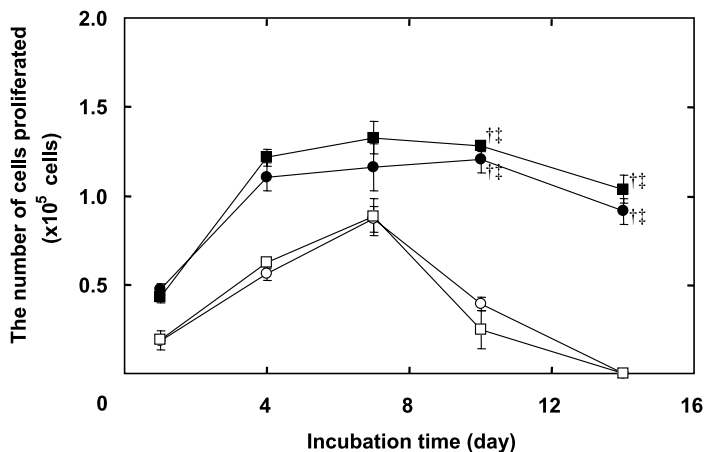


Figure 3. Proliferation profiles of MSC cultured with gelatin hydrogel microspheres with water contents of 98.7 (○), 96.3 (□), 93.5 (●) and 92.4% (■). † $P < 0.05$, significant against the number of cells cultured with gelatin hydrogel microspheres with a water content of 98.7% at the corresponding time. ‡ $P < 0.05$, significant against the number of cells cultured with gelatin hydrogel microspheres with a water content of 96.3% at the corresponding time.

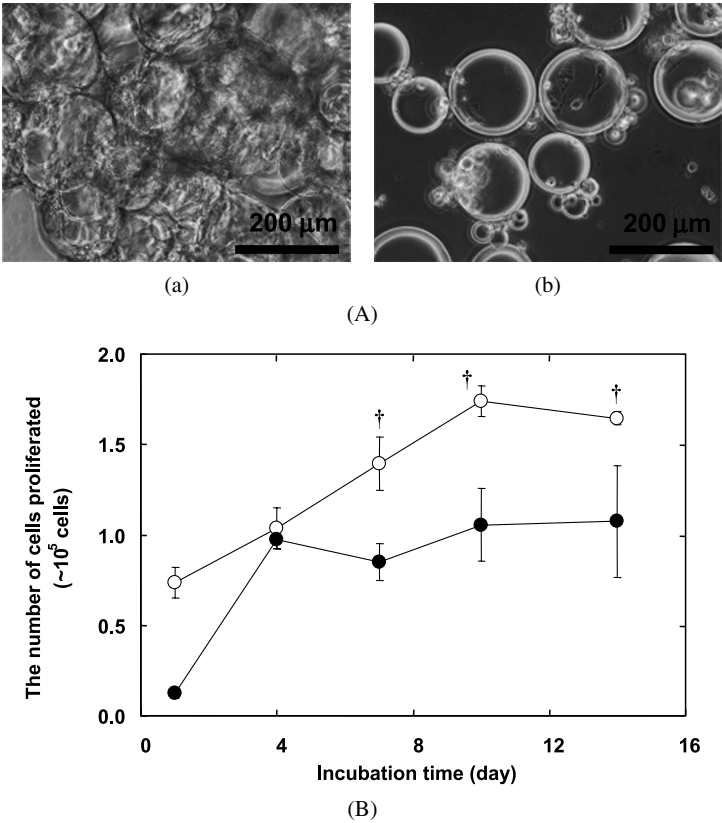


Figure 4. Proliferation of MSC cultured with gelatin hydrogel microspheres with a water content of 93.5 wt% at different densities. (A) Phase-contrast microscopic photographs of cells 14 days after culturing with gelatin hydrogel microspheres in one well of 48- (a) and 6-well multi-dish culture plates (b). (B) Time profiles of MSC proliferation in the presence of gelatin hydrogel microspheres at different densities for 48- (\circ) and 6-wells multi-well culture plate (\bullet). $\dagger P < 0.05$, significant against the number of cells cultured in a 6-well multi-dish culture plate at the corresponding time.

aggregated between the microspheres. However, cells were proliferated only on the surface of microspheres for a low density culture. Higher cell proliferation was observed in co-culturing with microspheres at the high density.

Figure 5 shows the comparison of cell proliferation profiles between gelatin hydrogel microspheres and sheets. Formation of cell aggregation was observed for culture with the microspheres. However, the cell aggregation was not formed on the hydrogel sheet. Better cells proliferation was detected for the microspheres culture while cell aggregation was also observed.

Figure 6 shows the viability of MSC proliferated with or without the gelatin hydrogel microspheres. The cells were aggregated in culture with the microspheres, in contrast to those on the plate. Since the MTT activity of cells is related to their viability, the cell viability for the hydrogel microspheres culture was significantly high compared with that of the hydrogel sheets.

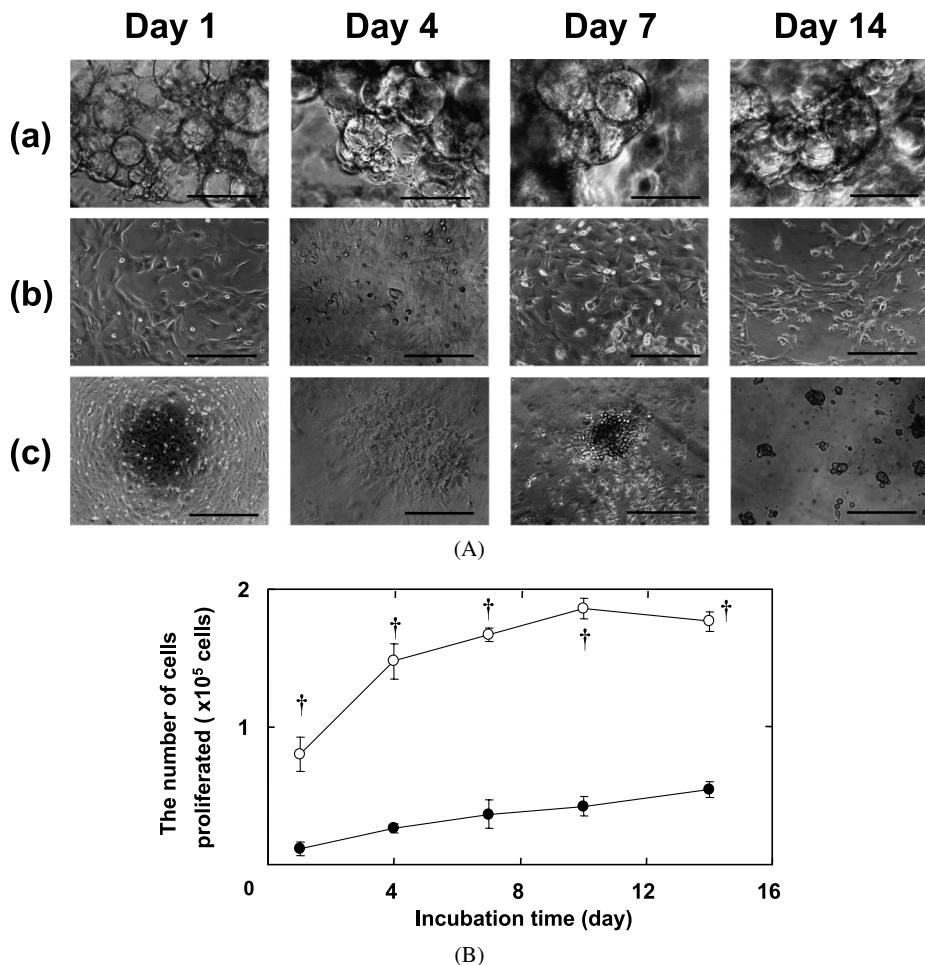


Figure 5. Proliferation of MSC cultured with gelatin hydrogel microspheres and sheets with a water content of 93.5 wt%. (A) Phase-contrast microscopic photographs of cells 1, 3, 7 and 14 days after culturing gelatin hydrogel microspheres (a) and sheets (b) or on the culture plate (c). (B) Time profiles of MSC proliferation with gelatin hydrogel microspheres (○) and sheets (●). † $P < 0.05$, significant against the number of cells cultured with gelatin hydrogel sheets at the corresponding time.

3.3. Production of sGAG from MSC Cultured with Gelatin Hydrogel Microspheres with or without TGF- β 1 Incorporation

Figure 7 shows the sGAG production of MSC cultured with the gelatin hydrogel microspheres and sheets in the differentiation medium. Irrespective of the incubation time, the amount of sGAG produced was significantly higher for cells cultured with the microspheres compared with that of culture on the hydrogel sheet and cell-culture plate. The amount increased with incubation time period.

Figure 8 shows the sGAG production of MSC cultured with the gelatin hydrogel microspheres incorporating TGF- β 1. The single addition of TGF- β 1 into the

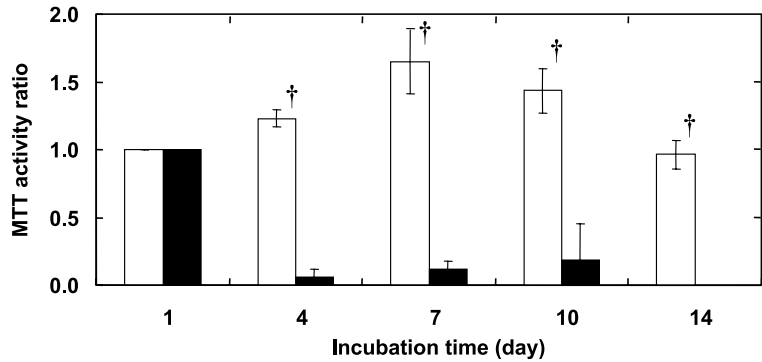


Figure 6. Viability of MSC cultured with gelatin hydrogel microspheres (□) and sheets (■) with a water content of 93.5 wt%. The activity ratio is expressed as the MTT activity of cells 1 day after culturing with gelatin hydrogel microspheres with 93.5% water content. † $P < 0.05$, significant against the MTT activity ratio of cells cultured with gelatin hydrogel sheets at the corresponding time.

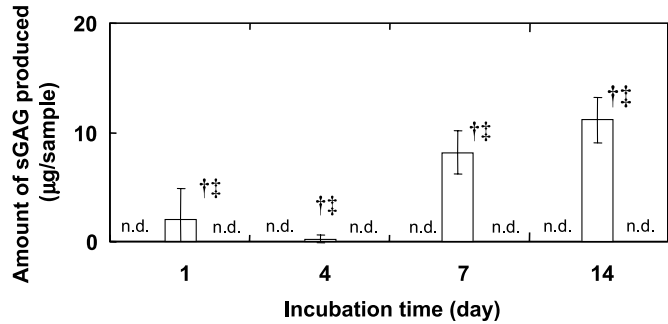


Figure 7. Time profiles of sGAG production from MSC cultured on the culture plate (□) or with gelatin hydrogel microspheres (◐) and sheets (■) with a water content of 93.5 wt% in the chondrogenic differentiation medium. n.d., not detected.

medium was not effective in enhancing the sGAG production. However, 8 times addition of TGF- β 1 promoted the production to a significantly higher extent. For the culture of MSC with the microspheres incorporating TGF- β 1, the similar significant promotion of sGAG production was observed. The incorporation of TGF- β 1 into the microspheres enhanced the sGAG production from MSC cultured in the differentiation medium.

4. Discussion

The present study indicates that the gelatin hydrogel microspheres functioned well as the scaffold of MSC proliferation. In addition, the TGF- β 1 release from the microspheres of cell scaffold accelerated the cell differentiation. Considering the extracellular matrix (ECM) of natural scaffold, there are two functions; the platform

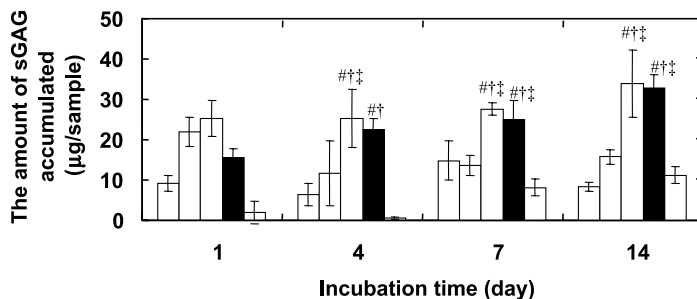


Figure 8. The amount of sGAG produced 4, 7 and 14 days after incubation with or without gelatin hydrogel microspheres incorporating TGF- β 1. MSCs were cultured in the chondrogenic differentiation (□, ▨, ▨, ■ and □). The medium does not contain TGF- β 1 (□) or does 10 ng/ml TGF- β 1 (⋯) which was added once on day 0, 1.25 ng/ml of TGF- β 1 (▨) which was added 8 times on day 0, 2, 4, 6, 8, 10, 12 and 14, and microspheres containing 10 ng of TGF- β 1 (■) or TGF- β 1-free, empty microspheres (□). # $P < 0.05$, significant against the amount of sGAG produced from MSC cultured at the corresponding time. † $P < 0.05$, significant against the amount of sGAG produced from MSC cultured in the chondrogenic differentiation medium without TGF- β 1 at the corresponding time. ‡ $P < 0.05$, significant against the amount of sGAG produced from MSC cultured in the chondrogenic differentiation medium which contains TGF- β 1 added once on day 0 at the corresponding time.

of cell proliferation, the so-called classical scaffold and the matrix of growth factor release. Many studies have been reported on the cell scaffold [16–18]. However, the aim of most studies was to design and prepare the classical scaffold which functions just as the cell platform. In this study, the microsphere scaffold was prepared from biodegradable and cytocompatible gelatin. MSCs proliferated initially on the surface of microspheres and later did between the multiple microspheres (Fig. 3). In addition, the gelatin microspheres functions as the matrix of growth factor release. We have demonstrated that the microspheres could achieve the controlled release of bioactive growth factors and succeeded in the growth-factor-induced regeneration of various tissues [13–17]. In Fig. 2, only about 15% TGF- β 1 was released from the microspheres. This is because the release test was performed in PBS where the microspheres are not degraded. For this hydrogel system, TGF- β 1 immobilized in the hydrogel through the physicochemical interaction between the TGF- β 1 and gelatin molecules can be released from the hydrogel only when the hydrogel is degraded to generate water-soluble gelatin fragments. It is possible that when cultured with the microspheres containing TGF- β 1, MSC secrete enzymes which degrade the microspheres to release TGF- β 1. TGF- β 1 released would act on MSC to effectively promote their chondrogenic differentiation (Fig. 8). Taken together, the microsphere is a cell scaffold which has physiological functions close to the natural ECM and is considered to be of next generation. It has been demonstrated that the MSC preparation and differentiation were accelerated by their combination with gelatin microspheres containing TGF- β 3 [18]. However, a pellet culture to combine the cells and microspheres was used while the type of TGF was different from that of the present study. In this study, the cell aggregate including

gelatin microspheres incorporating TGF- β 1 was formed by the co-culture system. The physiological formation of cells microspheres aggregates was observed based on the natural potential of MSC themselves for self aggregation.

The dependence of cell proliferation on the water content of microspheres can be explained in terms of microsphere degradation. The microspheres are degraded faster as the water content becomes higher. It is possible that MSC secrete some enzymes in culture, resulting in the enzymatic degradation of microspheres. When the microspheres scaffold is degraded during MSC culture, the substrate necessary for cell proliferation disappears and consequently cells would die, because MSC cannot proliferate without any substrates to attach.

MSC formed the cell aggregation although the extent depended on the density of scaffold microspheres (Fig. 4). First, cells proliferate on the surface of microspheres. At the high density of microspheres, the microsphere scaffold with cells attaching on the surface will contact to each other more frequently. It is likely that three-dimensional contact on the surface between different microspheres enables cells to interact and physiologically proliferate maintaining the natural interaction between cells in the in-between space of multiple microspheres, resulting in the formation of cell aggregates with formation of a physiological interaction network between cells. It is demonstrated that cells in the aggregated form show higher and better functions than those in the single form. Such natural interaction and proliferation of cells through their aggregation will be able to form the microenvironment of cells which is physiologically closer to that of ECM.

Neither strong MSC proliferation nor cell aggregation was observed on the hydrogel sheet (Figs 5 and 6). Cells, although slowly, proliferated to form their aggregates but the number and size were small compared with those of culture with the microspheres.

It is conceivable that in the two-dimensional system, even though the cell aggregates started to form, they could not interact with other cell aggregates in the three-dimensional fashion which is different from the case of microspheres culture. As the result, it is possible that the cell–cell interaction was so poor that the subsequent cell differentiation was not promoted (Fig. 7).

TGF- β 1 release was effective in enhancing the chondrogenic differentiation of MSCs (Fig. 8). One single addition of TGF- β 1 into the culture medium was not effective, but multiple addition (8 times) was effective. This clearly indicates the necessity of sustained TGF- β 1 supply for cell differentiation. It is highly conceivable that the gelatin microspheres can release TGF- β 1 during the cell culture, resulting in promoted cell differentiation by the mechanism of sustained TGF- β 1 supply.

The present study demonstrates that the scaffold of a microsphere type which can release TGF- β 1 functions well for the *in vitro* chondrogenesis of stem cells. The feasibility of *in vivo* activity is under investigation at present.

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